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# Evaluation of the Heterotrophic Plate Count Test for Drinking Water Safety: Comparing Culture-based vs. Molecular Methods for Identifying Bacteria

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**Abstract:**

Federal regulations of drinking-water quality regarding microbial contamination require the use of the culture-based heterotrophic plate count (HPC). This study compares HPC to culture-independent techniques for enumeration and identification of bacteria in environmental samples. Drinking water samples were collected from four different sites at two different times of year and analyzed using HPC, bacterial identification from 16s ribosomal RNA gene sequences, and direct cell counts from epifluorescence microscopy. No significant correlation was found between HPC measurements and either direct cell counts or measures of sample biodiversity from sequence analysis. 16s rRNA gene sequences from bulk DNA extractions reveal microbial communities in drinking water to comprise a broad array of bacterial diversity, including microbes of potential concern to human health such as mycobacteria. Conversely, HPC consistently selected for members of the Alphaproteobacteria (Sphingomonas, 45.8%; Methylobacteria, 33.8%; Porphyrobacter, 11.7%). These organisms comprised 25.0, 2.8, and 0.8 percent respectively of all 16s rRNA gene libraries from bulk DNA extractions. This result suggests that the heterotrophic plate count is not a relevant measure of drinking water quality.

**Introduction:**

The United States suffers an estimated 19.5 million cases of waterborne illness each year[1]. In approximately 47 percent of cases the etiology of the disease is unknown[1,2]. Despite this stark uncertainty in the actual quality of American drinking water, federally mandated tests of drinking water for microbial contamination rely largely on culture-based methods for microbial detection and assumptions about microbial ecology that have progressed little in the past century[3]. Though molecular, DNA-based methods currently exist to reliably and accurately identify the microbial constituents of environmental samples, information on the actual microbial consortia inhabiting water systems is lacking.

Municipal water utilities in the United States are required to evaluate the risk of microbial contamination for drinking water pursuant to the Total Coliform Rule (TCR). This regulation requires municipalities to monitor for the presence of coliforms (bacteria often found in mammalian digestive tracts) at end-user locations throughout the water distribution system[4]. The absence of coliforms is the main indicator for determining whether water is safe for human consumption. The Heterotrophic Plate Count (HPC) is used as an ancillary indicator of the accuracy of total coliform tests and is used by water monitors to provide data about water quality by approximating the levels of live heterotrophic bacteria in a water sample[5]. However, the HPC is severely limited in the range and precision of information the test provides, because the bacteria detected by the test are not generally pathogenic, and the test does not detect all pathogenic bacteria[6]. Furthermore, the methods of disinfection used by treatment facilities (primarily chlorination and chloramination) often allow the growth of some pathogens not detected by total coliform and HPC assays, including *Legionella pneumophila* and many potentially pathogenic species of mycobacteria[7,8]. Furthermore, HPC bacteria have been shown

experimentally to inhibit the growth of *Legionella*, meaning low plate counts could potentially obfuscate their presence[9]. These organisms are now known to pose a public health risk to consumers[8], yet the prevalence of these organisms is not well understood due to the lack of monitoring regimes for a broader suite of micro-organisms.

The Heterotrophic Plate Count is conducted by incubating a filtered sample of water on R2A agar plates, and counting number of resultant bacterial colonies that grow on each plate[5]. The number of colonies on a plate divided by the volume of water filtered onto that plate yields the number of colony forming units (CFU's) per milliliter. If CFU/mL exceeds 500 in a sample with a low chlorine residual level, the total coliform test may be indeterminate[5]. Given the selective properties of the R2A medium, colonies are assumed to consist of hetertrophic enteric bacteria (bacteria often found in mammalian digestive tracts, potentially indicative of fecal contamination), and bacterial colonies are often vaguely identified as specific species based on morphology[5]. However, advances in sequencing technologies now allow scientists to examine environmental microbial communities in much greater depth than was previously possible from culture-dependent techniques, such as those used in HPC[10].

Earlier understanding of microbiology relied on the study of microbes grown in culture. However, less than one percent of known microbial species can be induced to grow in culture[10]. Instead of examining a microbe in enrichment culture, we can now infer the identity of a microbe in an environmental sample by the sequences of its ribosomal RNA (rRNA) genes. The ribosome is found in all living organisms, and the genetic sequences that encode rRNA are relatively highly conserved among the three domains of life, making rRNA gene sequences ideal for determining broad-scale microbial phylogeny and genetic relatedness[10].

Though rRNA gene sequencing has been applied in many environmental contexts, the microbiology of drinking water is still relatively poorly characterized. Several studies have utilized rRNA gene sequencing to examine microbial communities in drinking water[11,7,12]. Kim Ross (Pace Laboratory, University of Colorado, Bouder) is currently conducting numerous surveys of the microbial ecology of drinking water system around the United States (unpublished data, personal communications, 2010-2011). These studies indicate that drinking water ecosystems are much more microbiologically complex than culture based surveys would suggest, and often contain potential pathogens not detected by standard water quality tests.

Several studies have compared HPC to both other culture-dependent and culture-independent techniques for examination of microbial ecosystems in drinking water. Carter et al. conducted HPC while monitoring physical and chemical properties of various water distribution systems and found HPC results to be uncorrelated with any chemical or physical parameter of water quality[13]. Hoefel et al. used culture-independent techniques to specifically monitor the effect of chloramine disinfection of water on the population of nitrifying bacteria and found that culture independent techniques revealed bacterial populations to be several orders of magnitude higher than indicated by HPC[11]. Lavender and Kinzelman compared agar-based enumeration of *Escherichia coli* in water samples to detection by quantitative polymerase chain reaction (qPCR), wherein an *E. coli*-specific gene was amplified to quantify the *E. coli* in the sample, and found qPCR to be more sensitive than culture-based methods[14].

Several studies have also attempted to correlate heterotrophic plate counts with measures of biodiversity from 16s rRNA gene analysis[15-17]. Burtscher et al. and Farleitner et al. both compared HPC results to denaturation-gradient gel electrophoresis (DGGE) profiles of 16s rRNA gene sequences in drinking water samples[15,17]. DGGE analysis is conducted by running

amplicons of 16s rRNA genes on a denaturation gel containing the denaturant in an increasing concentration gradient. Different DNA sequences will denature at different concentrations thus providing a fingerprint of the diversity of different sequences in the sample[15,17]. In some cases, DNA bands can be removed from the gel, purified, and sequenced to provide some insight into the specific microbial constituents of the sample[15]. Burtscher et al. conducted DGGE analysis on both DNA extractions from drinking water samples and on colonies of HPC plates from those same samples and did not find any of the same bands in either analysis, indicating differences in the make-ups of microbial communities as shown by DGGE and HPC. These results, however, are limited in the strength of their implications. Burtscher et al. sequenced the variable V3 region of several of the 16s rRNA bands of both the HPC and DNA extraction DGGE gels to give some idea of the specific organisms living in the water samples and isolated by HPC. However, these identifications are limited in phylogenetic accuracy based on the small sequence lengths compared to the entire 16s rRNA gene. Furthermore, the sequences represent only several samples of a community of thousands of organisms. At most, DGGE provides a general idea of the diversity of a sample. However, ecological fingerprinting methods such as DGGE cannot give as reliable statistical measures of biodiversity as full SSU rRNA gene sequencing studies, because fingerprints ignore the less abundant organisms that full phylogenetic analysis is capable of detecting[17]. In order to accurately evaluate the capabilities and limitations of the heterotrophic plate count, more precise molecular techniques for enumeration and identification of microbes must be employed.

Despite consistent findings that heterotrophic plate counts are not correlated with other measures of drinking water quality, the method is still widely used[18]. One reason for HPC's persistence is that high HPC observations (between 500 and 100 CFU/mL's) have been shown to

interfere with common, lactose-based, culture methods for detection of coliforms, and the total coliform rule is the primary measure of microbial contamination of drinking water in the United States[17]. However, it is unclear that the presence of coliforms (such as *E. coli*) is the primary concern in evaluating drinking water for human-health risks; Payment et al. demonstrated that tap-water in compliance with federal regulations was still 14-40% more likely to cause gastrointestinal disease than purified water, or treated tap water that is constantly purged[19]. The results of this study imply that there are other potential pathogens in United States' tap water not detected by current monitoring techniques. This highlights the dire need to understand how the heterotrophic plate count relates to the actual microbial biodiversity of drinking water sample—an understanding that can only be illuminated through 16s rRNA gene sequencing.

This study attempts to answer the above questions regarding HPC's relationship to actual microbial diversity, specifically with respect to microbes of potential human concern (e.g. *Mycobacteria*, *Legionellas*, etc.). This is accomplished in two primary ways. The bacterial constituents of drinking water samples were identified from 16s rRNA gene sequences and qualitatively compared to the results of HPC. Correlation coefficients relating HPC measurements to direct cell counts and to measures of biodiversity were calculated to quantitatively compare HPC to other parameters of water quality. It seems likely, given previous studies that found no correlation between HPC results and other parameters of drinking water quality--and the general imprecision of the HPC technique--that no correlation will be found with regard to CFU/mL and biodiversity. However, it is also plausible to think that the two might negatively correlate, because more diverse samples would likely contain microbes that compete with the limited suite of heterotrophs that appear on HPC plates. Such a result would indicate that low heterotrophic plate counts imply the presence of microbes that are not detected by

current monitoring techniques but which might be of potential human concern. Both hypotheses will be tested.

**Methods:**

*Sample Collection:*

Samples were collected from end-user locations and from the wastewater treatment plant around the area of Boulder, Colorado. Samples were collected by running taps until the chlorine residual of the water reached a value greater than 0.7 mg/L, then capturing 1 L of the sample in sterile containers for HPC and DNA extraction. Forty-five mL of water in a 50 mL conical tube containing 5 mL of 37% formaldehyde for epifluorescent staining and direct cell counting. Samples were collected from the same sites in July, 2010 and February, 2011. Samples collected in July were stored on ice until being returned to the lab. In order to maximize viability of bacteria in samples collected in February, samples were not stored on ice during transit.

*Heterotrophic Plate Counts:*

Heterotrophic plate counts were conducted according to protocols outlined in *Standard methods for the examination of water and wastewater*[20]: water samples were diluted in sterile buffered dilution water (Hatch Company, Dusseldorf, Germany) and filtered through white-gridded, 0.45 μm, 47 mm diameter Millipore membrane filters (EZ-Pak Membrane Filters, Millipore, Molsheim, France). The filters were then placed on R2A agar plates and incubated for 7 days at 29°C. The number of colonies on each plate was then counted according to the color and morphology of each colony.

Plates were made from dilutions of 1, 2, and 3 mLs of samples collected in July. Samples were collected in December, and again plated using 1, 2, and 3 mL dilutions. However, insufficient colonies grew on the plates made from the December samples to provide relevant HPC data, requiring recollection of samples in February. Based on the low plate counts from December's samples, dilutions of 1, 10, 100, and 200 mL were filtered and plated for each sample collected in February.

*16s rRNA gene sequencing from HPC colonies:*

For each sample, I selected one plate that was representative of the diversity in colony-types present for a given sample. Several colonies of each type (color and morphology) were selected randomly from each chosen plate and picked into tris-EDTA (TE) buffer (10μL of TE were used for July samples and 30μL of TE for February samples, due to difficulties in obtaining reliable PCR product from July's samples). Picked colonies in TE were boiled for 15 minutes at 85°C and subsequently centrifuged at 3700 rpm for 10 minutes. One μL of supernatant was pipetted into a PCR reaction mixture with 5 Prime HotMasterMix (5 Prime, Inc., Hamburg, Germany) and 515 forward/1391 reverse universal small-subunit rRNA gene primers. The reaction mixtures then underwent 29 cycles of the polymerase chain reaction.

Amplified 16s rRNA genes from each colony were then aliquotted into two reactions and cleaned (to remove unincorporated nucleotides and other leftover reagents) using ExoSAP-IT (Affymetrix, Inc., Santa Clara, CA). Cleaned PCR product was prepared for sequencing using the DYEnamic ET Dye Terminator Kit (MegaBACE, GE Healthcare, Piscataway, NJ) as per manufacture instruction. Sequences were obtained using the Pace Laboratory's MegaBACE 96-well capillary sequencer.

*DNA extraction and amplification:*

Water samples were filtered through 0.2μm polycarbonate filters. Genomic DNA was then extracted from the filters using phenol-chloroform extraction and resuspended in TE. SSU rRNA genes from the DNA extractions were amplified using 515f and 1391r universal SSU rRNA gene primers and 5 Prime HotMasterMix (5 Prime, Inc., Hamburg, Germany).

*SSU rRNA gene cloning:*

Amplified SSU rRNA genes from the DNA extractions were gel-purified. Gel purification allows separation of eukaryotic 18s rRNA genes from bacterial and archeal 16s rRNA genes. However, as sequences obtained from DNA extractions were only to be compared with gene sequences from bacterial colonies picked from HPC plates, only 16s rRNA genes were cloned for the purpose of this study (that is, the smaller (16S) band was excised and purified and the 18S band was not used). Purified DNA was incorporated into PCR4-TOPO vector (Invitrogen Corp.) containing ampicillin-resistance genes and T3/T7 primer sites. Vectors containing the incorporated 16s rRNA genes were cloned into electrocompetent TOPO-4 *Esherichia coli* cells using electroporation. Transfected cells were incubated overnight on LB-agar/ampicillin plates.

*16s rRNA gene sequencing from DNA-extraction amplicons:*

Cloned colonies were picked, regrown, and boiled in TE. Supernatant from boiled biomass underwent T3/T7 PCR. PCR product was cleaned using ExoSAP-IT. Cleaned PCR product was prepared for sequencing using DYEnamic ET Dye Terminator Kit. 16s rRNA gene

sequences were obtained for each sample using the Pace Laborotory's MegaBACE 96-well capillary sequencer.

*Sequence Analysis:*

16s rRNA gene sequences from both HPC colonies and from bulk DNA extraction clone libraries were BLASTed against the all-species Living Tree Project 16s rRNA gene database for named species[21] using XplorSeq[22]. BLASTed sequences were then aligned using SILVA's SINA online SSU rRNA gene aligner[23]. Aligned sequences were inserted into SILVA's SSU reference database 104 using ARB's parsimony insertion function[24]. Phylogenetic lineage information about each sequence was then exported from ARB for bacterial identification and biodiversity calculations.

*Direct cell counts:*

Water samples were fixed at 3.7% formaldehyde. Samples were filtered through 0.2μm black polycarbonate 25mm diameter filters. Filters were mounted on glass microscope slides using citifluor mounting agent. 0.5 mL of 2.5 μg/mL 4'-6-diamidino-2-phenylidole (DAPI)--diluted in pH 7.8 10 mM filter-sterilized tris buffer--was spotted onto each filter. Each filter was allowed to stain for 5 minutes then cleaned with filter-sterilized tris. Cells were then visualized and counted at 40x magnification using epifluorescence microscopy. Counts per field of view were multiplied by the area of each filter over the area of each field then divided by the volume of sample filtered ((count x (area of filter / area of field)) / volume), then averaged for each sample to obtain counts in cells/mL for each sample.

**Results:**

Samples were collected from four sites. Three of the four sites (HS, C1, and MC) were collected from public end-user taps around Boulder, Colorado. The remaining sample (WW) was collected from the outflow of a wastewater treatment plant. Samples were collected from these same four sites in July and December of 2010, and February of 2011, then processed as per the procedures outlined above. Samples collected in December did not yield results sufficient for data analysis and discussion. The remainder of this paper will focus on data obtained from samples collected from the four aforementioned sites in July and February.

*Heterotrophic Plate Counts:*

Multiple dilutions (from different volumes) were plated of each sample. The heterotrophic plate count data presented here represents the mean CFU/mL calculated from all plates for each sample. If the number of colonies that grows on a plate is not linearly related to the volume of water that was filtered for that plate, it is plausible to think that the volume filtered will influence the CFU/mL calculation for that plate. This would suggest that averaging across plates with different volumes filtered for the same sample may not accurately represent the CFU/mL level of that sample. To test for this a correlation coefficient was calculated for all CFU/mL calculations across all volumes-filtered and all samples. The correlation coefficient was calculated as -0.243 with a p-value of 0.0829 (degrees of freedom = 32). This test indicates that there is no significant correlation (at a 95% confidence level) between CFU/mL calculated for a specific plate, and the volume of water filtered for that plate, lending credibility to the procedure of averaging across plates for the same sample.

**Figure 1** shows the average CFU/mL calculated for each sample from both collections (July and February). The average CFU/mL for all eight samples was 18.8. Plate counts of the samples collected in July yielded and average CFU/mL of 34.5, whereas samples collected in February averaged only 3.5 CFU/mL.

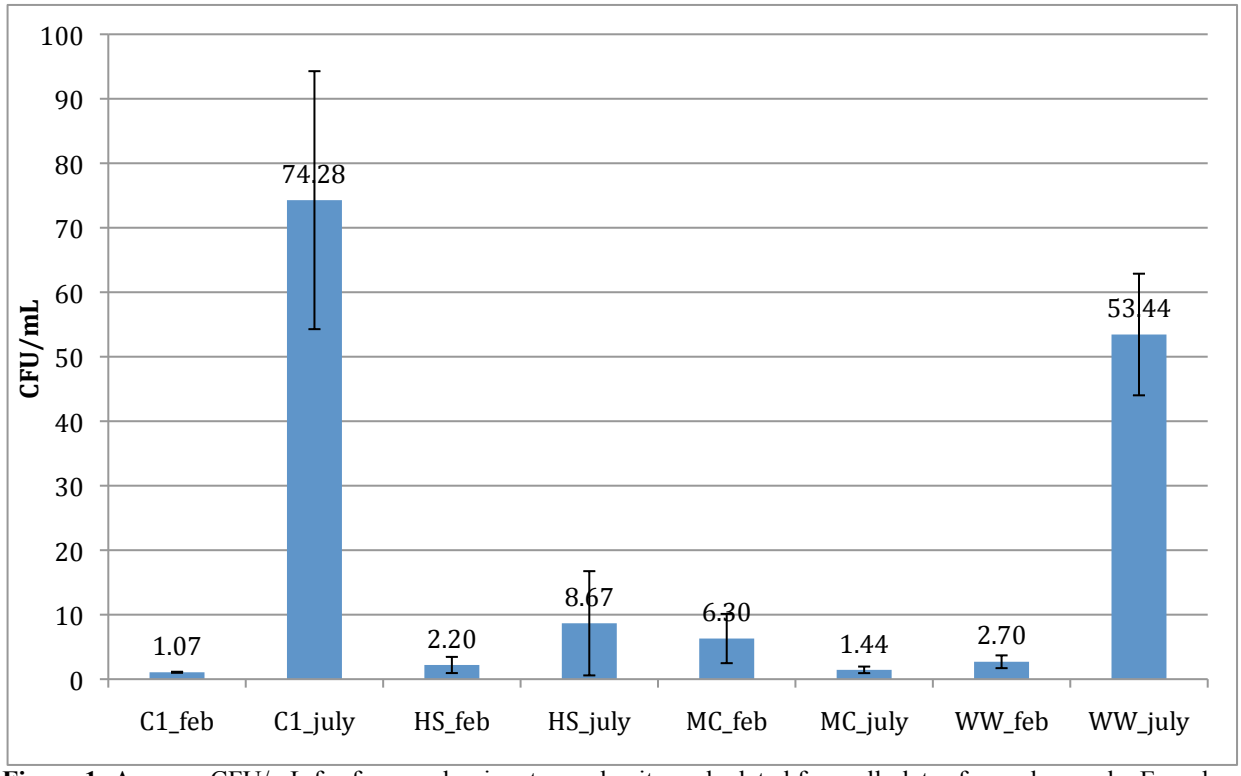



**Figure 1:** Average CFU/mL for four predominant sample-sites calculated from all plates for each sample. Error bars represent standard deviation.

*Direct Cell Counts:*

The average cell count across all eight samples was on the order of $10^3$ cells/mL. On average, samples collected in July had an order of magnitude more cells/mL than samples collected in February with significant differences in average cell counts for samples collected in July and February ($p = 5.058 \times 10^{-7}$, $df = 113$). Cell counts ranged from $10^2$ cells/mL to $10^3$ cells/mL. This range corroborates expectations of cell concentrations in drinking water from previous studies; Hoefel et al. compared the enumeration of bacteria in drinking water in

Southern Australia using HPC and flow cytometry (staining samples with propidium iodide/Syto 9) and found both that cell counts from flow cytometry consistently ranged from $10^2$ to $10^4$ cells/mL and were 1-2 log units higher than CFU/mL from HPC[24,25].

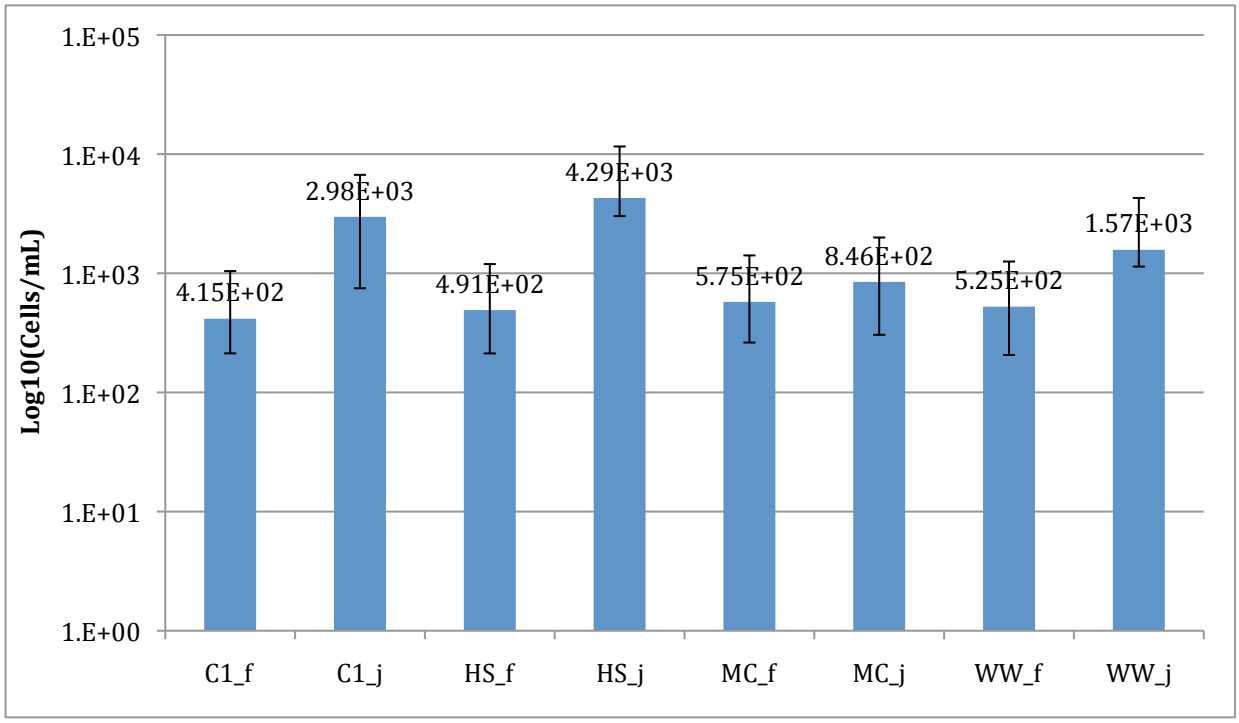



**Figure 2:** Direct cell counts (cells/mL on log scale) of eight primary samples. Error bars represent 95% confidence intervals under the assumption that the data follows a t-distribution.

Nevertheless, the precision of the data is limited by several elements of the experiment's design. Several counts were performed over the course of the study on each sample by filtering only part of the sample each time. The cell counts in cells/mL reported here represent the mean of all counts for each sample. Nested analysis of variance was performed to test whether there was significant variance in each sample's average cell count between each time that the sample was counted, the results of which are summarized in **table 1**.

| Source of Variation | Degrees of Freedom | Sum of Squares | Mean Squares | F Ratio | P(F) |
|---|---|---|---|---|---|
| Sample | 8 | 134546250 | 16818281 | 5.606656 | 0.01741311 |
| Count | 7 | 20997892 | 16818281 | 1.952968 | 0.06916349 |
| Error | 99 | 152060956 | 1535969 | | |

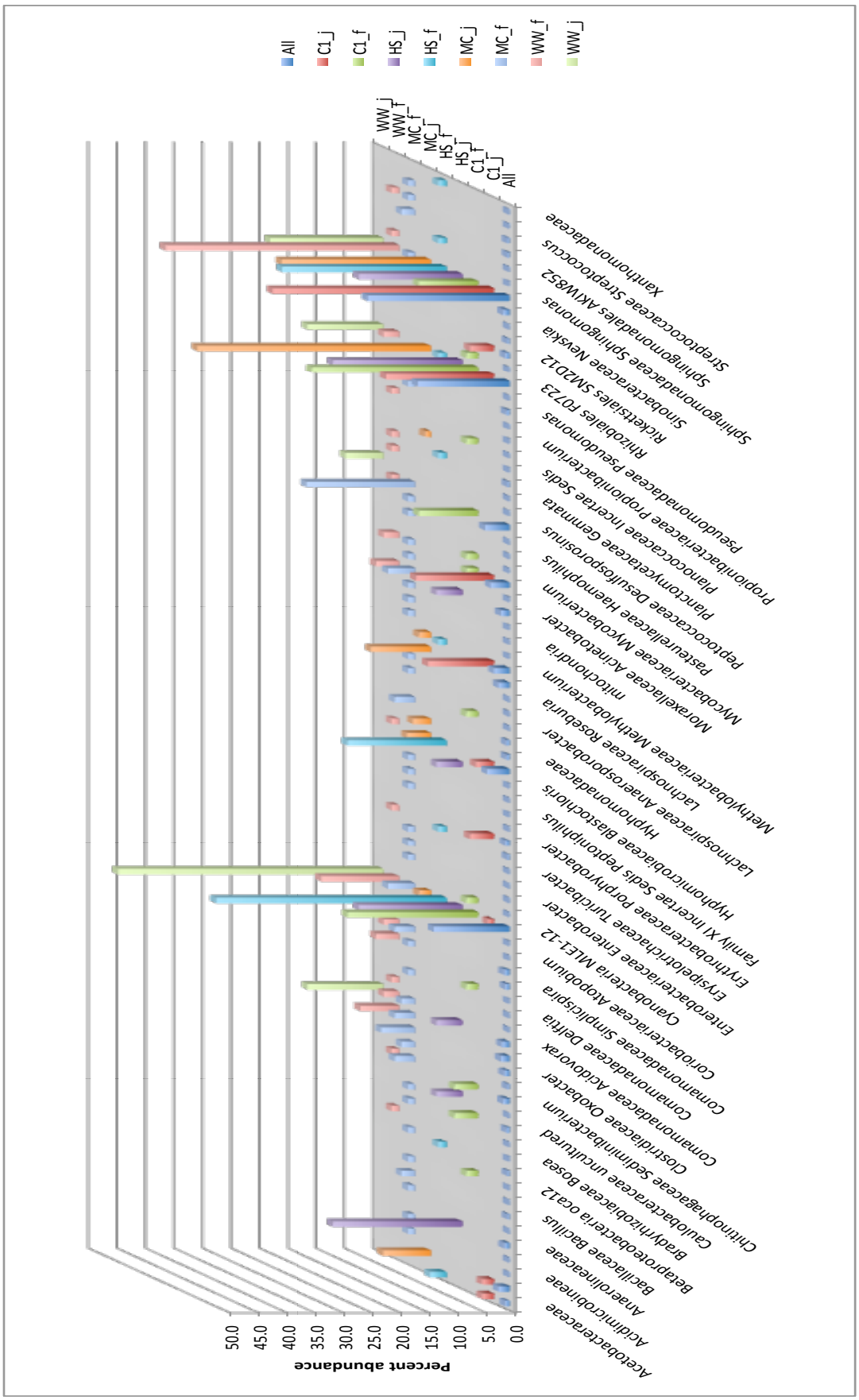
**Table 1:** Nested analysis of variance in cell count data between samples and within each sample between counts.

This test shows that, although there is significant variation in the mean cell counts between samples, the variation in each sample's average from the different preparations (i.e. each time the sample was aliquoted and counted) is not significant. This allows the legitimate averaging of cell counts for each sample across preparations, which in turn enables the performance of the model-I, 1-way analysis of variance above, comparing averages from the same collection site across collection times.

*16s rRNA Gene Sequences:*

Sequences from bulk DNA extractions:

**Figure 4** summarizes the relative abundance of bacteria identified by phylogenetic analysis from the DNA libraries of all eight samples. The predominant groups in most samples were Sphingomonadaceae, Cyanobacteria, and Rhizobiales. General patterns of relative abundance within the bacterial libraries appear to be conserved across samples, and even more strongly within the same samples collected at different times.


All
C1_j
C1_f
HS_j
HS_f
MC_j
MC_f
WW_f
WW_j
Percent abundance
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
Acetobacteraceae
Acidimicrobiinae
Anaerolineaceae
Bacillaceae Bacillus
Betaproteobacteria
Bradyrhizobiaceae
Caulobacteraceae Bosea
Chitinophagaceae Sediminibacterium
Clostridiaceae uncultured
Comamonadaceae Oxobacter
Comamonadaceae Acidovorax
Comamonadaceae Delftia
Corynebacteriaceae Simplicispira
Cyanobacteria Acaryochloris
Enterobacteriaceae MLE1-12
Erysipelotrichaceae Turicibacter
Family XI Incertae Sedis Peptoniphilus
Hyphomicrobiaceae Blastochloris
Hyphomonadaceae
Lachnospiraceae Anaerosporobacter
Lachnospiraceae Roseburia
Methylobacteriaceae Methylobacterium
Mitochondria
Moraxellaceae Acinetobacter
Mycobacteriaceae Mycobacterium
Pasteurellaceae Haemophilus
Peptococcaceae Desulfosporosinus
Planctomycetaceae Gemmata
Planococcaceae Incertae Sedis
Propionibacteriaceae Propionibacterium
Pseudomonadaceae Pseudomonas
Rhizobiales F0723
Rickettsiales SM2D12
Sinobacteraceae Nevskia
Sphingomonadaceae Sphingomonas
Sphingomonadales AKIW852
Streptococcaceae Streptococcus
Xanthomonadaceae

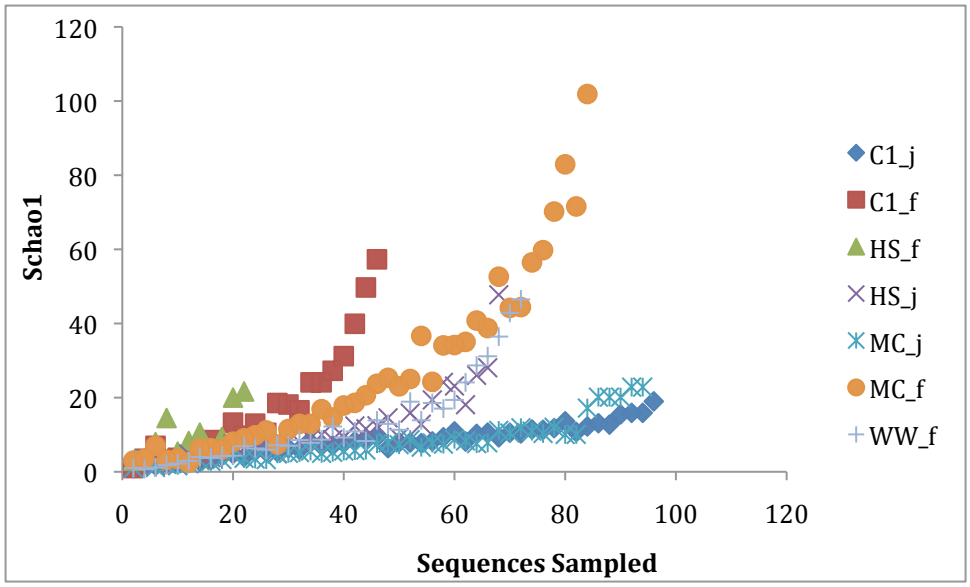



**Figure 5:** $S_{Chao1}$ vs sequences sampled for different numbers of sequences (clustered at 97% sequence identity) from all samples except WW_j; because of the relatively low number of observed sequences, almost all sequences observed in WW_j's library were observed only once, causing the $S_{Chao1}$ measure for WW_j to be an order of magnitude higher than for any other sample.

**Figure 5** shows the predicted diversity in each sample, estimated by $S_{Chao1}$. There does appear to be a difference in predicted diversity between the libraries, although this may reflect the small size of the libraries: because of the relatively low number of observed sequences, almost all sequences observed in WW_j's library were observed only once, causing the $S_{Chao1}$ measure for WW_j to be an order of magnitude higher than for any other sample. $S_{Chao1}$ is calculated from the total number of species observed in a sample plus the square of the number of species observed only once, divided by twice the number of species observed two times[26]. $S_{Chao1}$ thereby provides an estimate of the diversity of organisms observed in a sample. Because total number of species observed and the number of species rarely observed increase with the number of samples taken until all species have been observed multiple times, $S_{Chao1}$ increases with sampling. Therefore, $S_{Chao1}$ also provides a measure of how thoroughly a sample has been sequenced; as the full range of biodiversity in a sample approaches complete observation, $S_{Chao1}$ of the sample no longer increases with increased sequences sampled. By this measure, it would

appear that none of the samples collected in this study have been sequenced fully enough to get a complete idea of the biodiversity contained within each sample.

Sequences from HPC plates:

Colonies on HPC plates were counted by color and morphology if obviously different colony structures existed within the same color-group (e.g. large rough versus small round yellow colonies). Colonies representative of each colony-type on each plate were picked for 16s rRNA gene sequencing to identify each colony's constituent bacteria. Sequences were categorized primarily by the sample from which they originated and the color of the colony they represented. All of the HPC-plate sequence data, aggregated by color and identified through phylogenetic analysis are presented in **table 2**.

| **Bacterial Lineage** | | | | | **Colony type** | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Phylum | Class | Order | Family | Genus | p | w | y | o | b |
| Firmicutes | Bacilli | Bacillales | Staphylococcaceae | Staphylococcus | 0 | 0 | 1 | 0 | 0 |
| Proteobacteria | Alphaproteobacteria | Rhizobiales | Methylobacteriaceae | Methylobacterium | 37 | 3 | 1 | 0 | 0 |
| Proteobacteria | Alphaproteobacteria | Rickettsiales | | | 0 | 0 | 1 | 0 | 0 |
| Proteobacteria | Alphaproteobacteria | Sphingomonadales | Erythrobacteraceae | Porphyrobacter | 1 | 0 | 1 | 5 | 5 |
| Proteobacteria | Alphaproteobacteria | Sphingomonadales | Erythrobacteraceae | | 0 | 0 | 1 | 0 | 0 |
| Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | Blastomonas | 0 | 0 | 11 | 0 | 0 |
| Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | Sphingomonas | 0 | 1 | 49 | 0 | 0 |
| Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | Sphingopyxis | 0 | 0 | 1 | 0 | 0 |
| Proteobacteria | Gammaproteobacteria | Xanthomonadales | Sinobacteraceae | | 0 | 0 | 0 | 0 | 1 |
| Proteobacteria | Gammaproteobacteria | Xanthomonadales | Sinobacteraceae | Nevskia | 0 | 0 | 0 | 0 | 18 |

**Table 2:** Phylogenetic identification of all HPC colonies classified by color. p=pink, w=white, y=yellow, o=orange, b=brown.

The pink colonies were most likely *Methylobacteria*. The white colonies were too small to get consistent PCR, so whichever wells were labeled as white more likely contained genetic material from other colonies and were therefore identified predominantly as species attributed to other colony-types; as a result, and because of their relative inabundance, the white colonies will be ignored for the remainder of the study. Yellow colonies were identified predominantly as two

different Sphingomonodacae bacteria, but there was no consistent pattern regarding which colonies were identified as each genus, so all yellow colonies will be treated as *Sphingomonas* for the purpose to this study. Orange colonies are assumed to be *Porphyrobacter* and all brown colonies are treated as *Nevskia*.

Comparison between HPC sequences and extraction sequences:

**Table 3** compares the relative abundance of bacterial species identified by phylogenetic analysis of sequences obtained from DNA extractions with the relative abundance of each species ascribed to each colony-type on each HPC plate, for each sample.

| | Total ext | Total HPC | C1_f ext | C1_f HPC | C1_j ext | C1_j HPC | HS_f ext | HS_f HPC | HS_j ext | HS_j HPC | MC_f ext | MC_f HPC | MC_j ext | MC_j HPC | WW_f ext | WW_f HPC | WW_j ext | WW_j HPC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sphingomonadaceae Sphingomonas | 25.0 | 45.8 | 10.4 | 32.2 | 38.9 | | 18.2 | 56.1 | 29.0 | 95.2 | 1.2 | 97.8 | 26.3 | 22.2 | 41.1 | 47.2 | 20.0 | 4.64 |
| Rhizobiales F0723 | 16.2 | | 29.2 | | 18.9 | | 22.7 | | 1.4 | | 0.0 | | 41.1 | | 2.7 | | 13.3 | |
| Cyanobacteria MLE1-12 | 13.4 | | 22.9 | | 1.1 | | 18.2 | | 40.6 | | 4.8 | | 2.1 | | 13.7 | | 46.7 | |
| Peptococcaceae Desulfosporosinus | 4.4 | | 10.4 | | 0.0 | | 0.0 | | 0.0 | | 19.0 | | 0.0 | | 1.4 | | 0.0 | |
| Hyphomicrobiaceae Hyphomicrobium | 4.0 | | 0.0 | | 3.2 | | 0.0 | | 17.4 | | 0.0 | | 4.2 | | 1.4 | | 0.0 | |
| Mycobacteriaceae Mycobacterium | 3.4 | | 2.1 | | 13.7 | | 0.0 | | 0.0 | | 1.2 | | 0.0 | | 2.7 | | 0.0 | |
| Methylobacteriaceae Methylobacterium | 2.8 | 33.8 | 0.0 | 52.5 | 11.6 | 49 | 0.0 | 43.1 | 1.4 | 4.76 | 0.0 | 2.22 | 2.1 | 55.6 | 0.0 | 52.8 | 0.0 | 1.99 |
| Acetobacteraceae Rhodovarius | 2.0 | | 0.0 | | 2.1 | | 0.0 | | 0.0 | | 0.0 | | 8.4 | | 0.0 | | 0.0 | |
| Methylobacteriaceae Meganema | 2.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 10.5 | | 0.0 | | 0.0 | |
| Comamonadaceae | 1.6 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 3.6 | | 0.0 | | 6.8 | | 0.0 | |
| Moraxellaceae Acinetobacter | 1.6 | | 0.0 | | 0.0 | | 4.5 | | 0.0 | | 4.8 | | 0.0 | | 4.1 | | 0.0 | |
| Comamonadaceae Acidovorax | 1.4 | | 0.0 | | 0.0 | | 4.5 | | 0.0 | | 2.4 | | 0.0 | | 2.7 | | 13.3 | |
| Chitinophagaceae Sediminibacterium | 1.2 | | 4.2 | | 0.0 | | 0.0 | | 0.0 | | 3.6 | | 0.0 | | 1.4 | | 0.0 | |
| Sphingomonadaceae Sphingobium | 1.2 | | 2.1 | | 0.0 | | 0.0 | | 0.0 | | 6.0 | | 0.0 | | 0.0 | | 0.0 | |
| Anaerolineaceae | 1.0 | | 0.0 | | 0.0 | | 22.7 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | |
| Comamonadaceae Variovorax | 1.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 3.6 | | 0.0 | | 2.7 | | 0.0 | |
| Clostridiaceae Oxobacter | 1.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 6.0 | | 0.0 | | 0.0 | | 0.0 | |
| Erythrobacteraceae Porphyrobacter | 0.8 | 11.7 | 0.0 | 14.6 | 4.2 | 49.5 | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | 26.5 |
| Rickettsiales SM2D12 | 0.8 | | 0.0 | | 4.2 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | |
| Acetobacteraceae | 0.8 | | 0.0 | | 2.1 | | 0.0 | | 2.9 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | |
| Comamonadaceae Simplicispira | 0.8 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 1.2 | | 0.0 | | 4.1 | | 0.0 | |
| Hyphomonadaceae | 0.6 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 3.2 | | 0.0 | | 0.0 | |
| Caulobacteraceae uncultured | 0.6 | | 4.2 | | 0.0 | | 0.0 | | 0.0 | | 1.2 | | 0.0 | | 0.0 | | 0.0 | |
| Pseudomonadaceae Pseudomonas | 0.6 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 4.1 | | 0.0 | |
| Lachnospiraceae | 0.6 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 3.6 | | 0.0 | | 0.0 | | 0.0 | |
| Erythrobacteraceae | 0.4 | | 0.0 | | 0.0 | | 0.0 | | 1.4 | | 0.0 | | 0.0 | | 1.4 | | 0.0 | |
| Planctomycetaceae Planctomyces | 0.4 | | 0.0 | | 0.0 | | 0.0 | | 1.4 | | 0.0 | | 0.0 | | 1.4 | | 0.0 | |
| Bradyrhizobiaceae | 0.4 | | 0.0 | | 0.0 | | 0.0 | | 1.4 | | 1.2 | | 0.0 | | 0.0 | | 0.0 | |
| Streptococcaceae Streptococcus | 0.4 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 1.2 | | 0.0 | | 1.4 | | 0.0 | |
| Bacillaceae Bacillus | 0.4 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 2.4 | | 0.0 | | 0.0 | | 0.0 | |
| Chloroplast | 0.4 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 2.4 | | 0.0 | | 0.0 | | 0.0 | |
| Ruminococcaceae Incertae Sedis | 0.4 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 2.4 | | 0.0 | | 0.0 | | 0.0 | |
| Staphylococcaceae Staphylococcus | 0.4 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 2.4 | | 0.0 | | 0.0 | | 0.0 | |
| Phyllobacteriaceae | 0.2 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 6.7 | |
| Sinobacteraceae Nevskia | 0.2 | 8.73 | 0.0 | | 0.0 | | 0.0 | 0.81 | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 6.7 | 66.9 |
| Chitinophagaceae | 0.2 | | 0.0 | | 0.0 | | 4.5 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | |
| Hyphomicrobiaceae Blastochloris | 0.2 | | 0.0 | | 0.0 | | 4.5 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | |

**Table 3:** Relative abundance of bacteria in bulk DNA extraction clone libraries and on HPC plates. Values in columns labeled "ext" represent the percentage of sequences in the clone library for that sample that were identified as a particular bacterial group. Values in columns labeled "HPC" represent the percentage of colonies on the HPC plates that were identified as belonging to a particular bacterial group.

*Sphingomonas* was the only bacterium generally common to sequences both from DNA extractions and plates. The species that predominated on HPC plates were rarely significant in the bacterial population as determined by 16s rRNA gene libraries for the same water samples. In particular, methylobacteria appeared to be much more dominant on HPC plates than in the actual bacterial communities.

The phylogenetic lineage information displayed above was obtained by inserting SINA-aligned sequences into SIVLA's SSU rRNA reference database using ARB's parsimony insertion function. Sequences were also aligned using BLAST against the Living Tree Project's non-environmental species 16s rRNA gene database, comprised primarily of organisms cultured in a clinical setting[22]. BLAST hits were not used for species identification. Interestingly, however, the average percent identity for HPC sequences BLASTed against the database was 97.2, whereas sequences obtained from clone libraries, on average, were matched to sequences in the database at 96.1 percent identity. Of the ten identified groups of sequences from all HPC plates, two were matched at 94 percent sequence identity and one at 95 percent; the remaining sequences all BLASTed at 98 or 99 percent sequence identity. Sequences from bulk DNA extraction ranged from 89 to 100 percent sequence identity.

*Correlation between cells/mL and CFU/mL:*

**Figure 6** shows a plot of the calculated cells/mL for all eight samples against CFU/mL for those same samples. The correlation coefficient was calculated as 0.4576731 with a p-value of 0.1508896 (n = 8), meaning the data did not show a significant correlation between cell counts and plate counts.

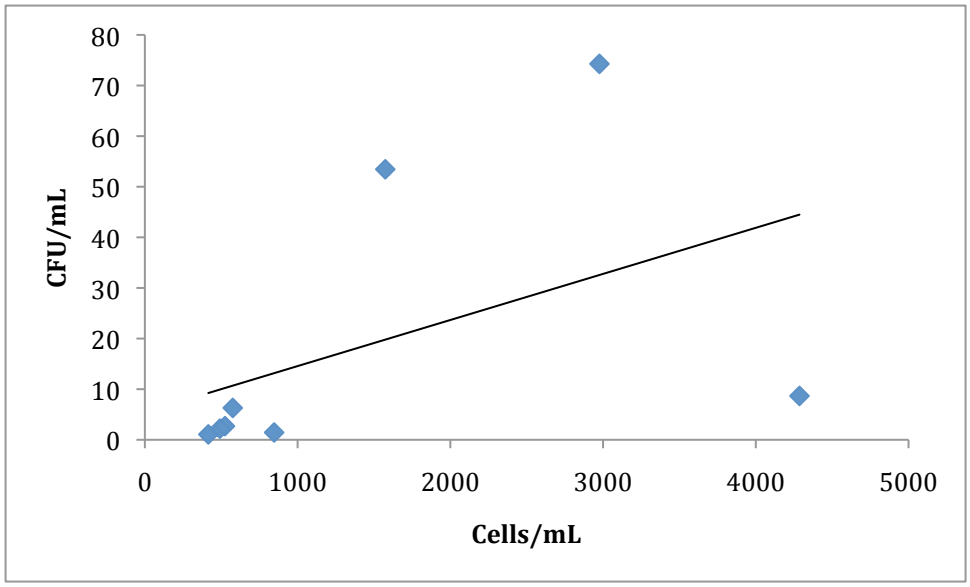



**Figure 6:** Scatterplot of CFU/mL vs Cells/mL for all samples.

However, the power of this estimate of correlation is 0.18 meaning there is a probability of 0.82 of not seeing a significant correlation when correlation is in fact present (power was calculated using Z transformations of the correlation coefficient[27]). In order to achieve a power of 0.80 for a similarly weak correlation, approximately 35 observations would be required.

*Correlation between biodiversity and CFU/mL:*

A correlation coefficient was also calculated relating CFU/mL of a sample calculated from plate counts to the biodiversity of a sample, estimated by $S_{Chao1}$. **Figure 7** summarizes the relationship between CFU/mL and $S_{Chao1}$ for seven of the eight samples (WW_j was excluded for reasons explained above). The correlation coefficient was calculated as 0.3076833 with p-value of 0.2510151 (n = 7), meaning the data was insufficient to observe any significant correlation between CFU/mL and $S_{Chao1}$. The power of this test was calculated to be 0.08. In order to achieve power of 0.80, approximately 80 observations would be required.

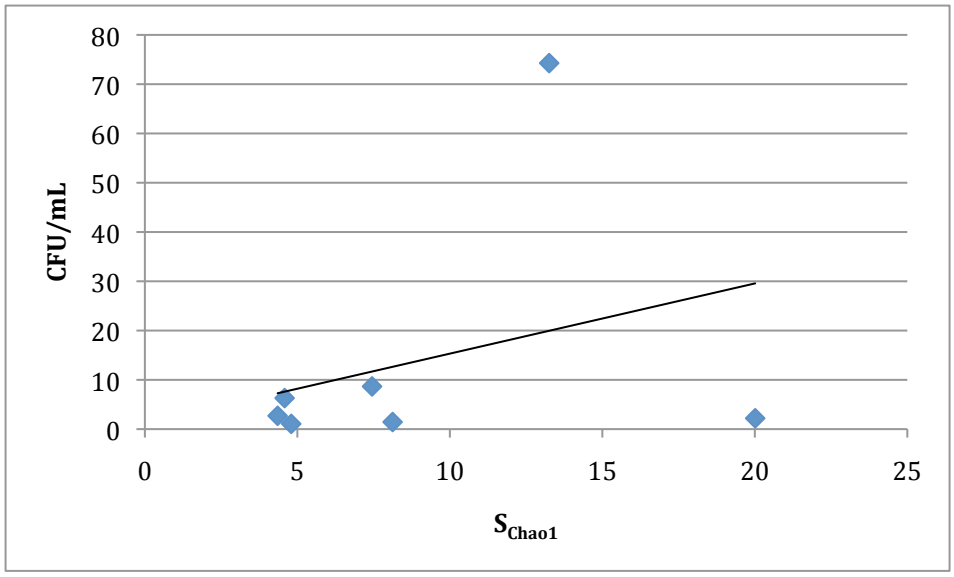



**Figure 7:** Scatterplot of CFU/mL vs $S_{Chao1}$ for all samples except WW_j.

**Discussion:**

The data collected in this study showed no significant correlation between heterotrophic plate counts and either direct cell counts, or relative biodiversity. Furthermore, side-by-side comparisons of the bacteria identified by 16s rRNA gene sequencing from bulk DNA extraction and heterotrophic plate counts reveals little similarity in overall community diversity as well as significant distortions in relative abundance, particularly for *Methylobacteria*. Together, these results imply that the heterotrophic plate count has little relevance for determining parameters of drinking water quality regarding microbial communities. This is particularly striking given that HPC may not detect the potential presence of microbes of concern to human health. In particular, this study found significant levels of *Mycobacteria* in the sample collected from C1 in July.

The genus mycobacterium is of particular concern for human health because of the diversity of obligate and opportunistic human pathogens contained within the genus[28]. The particular sequences identified as *Mycobacteria* in the July C1 sample were identified by BLAST as *Mycobacteria asiaticum* with 98% sequence identity, an average bit score of 1364, and

sequences of average length 744 base pairs. *M. asiaticum* has been identified as possible source of pulmonary diseases and infection[29]. However, given high conservation in mycobacterial 16s rRNA gene sequences, further analysis would need to be conducted before making any strong conclusions about the presence of possible mycobacterial pathogens in the C1 sample. Interestingly, the July C1 sample had the highest plate count of any of the eight samples processed for this study. However, the CFU/mL of the July C1 sample was still less than one fifth the amount set as the upper limit for acceptable drinking water in the United States.

The primary limitation of the data collected for this study was that the samples collected represent a small subset of the range in drinking water quality that is acceptable under federal regulations. Heterotrophic plate counts up to 500 CFU/mL are considered acceptable for human consumption under the Total Coliform Rule. However, HPCs of samples collected for this study were consistently observed to be less than 100. Estimates of correlation are known to be weaker over smaller ranges of data, meaning that even if a correlation could have been observed between HPC and biodiversity or bacterial load, it is unlikely that such correlation could have been accurately estimated given the scope of this study. It is difficult to conclude from the results of this study that the heterotrophic plate count is of no value as an indicator of drinking water quality. Instead, however, the results of this study suggest the strong need for further investigations into exactly what HPC measures regarding drinking water quality. Such investigations will require samples over a broader range of water qualities, as well as more robust estimates of total bacterial load and bacterial diversity within those samples.

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**References:**


1. Reynolds, K. a, Mena, K.D. & Gerba, C.P. Risk of waterborne illness via drinking water in the United States. *Reviews of environmental contamination and toxicology* **192**, 117-58(2008).
2. Craun, G.F. et al. Causes of Outbreaks Associated with Drinking Water in the United States from 1971 to 2006. *Clin. Microbiol. Rev.* **23**, 507-528(2010).
3. Allen, M.J., Edberg, S.C. & Reasoner, D.J. Heterotrophic plate count bacteria--what is their significance in drinking water? *International Journal of Food Microbiology* **92**, 265-274(2004).
4. Total Coliform Rule : A Quick Reference Guide. 2-3(1989).
5. Reasoner, D.J. Heterotrophic plate count methodology in the United States. *International Journal of Food Microbiology* **92**, 307-315(2004).
6. Bartram, J. *Heterotrophic plate counts and drinking-water safety: the significance of HPCs for water quality and human health*. 272(IWA Publishing: 2003).at <http://books.google.com/books?id=apqcbAN7QL4C>
7. Pryor, M. et al. Investigation of opportunistic pathogens in municipal drinking water under different supply and treatment regimes. *Water Science and Technology: A Journal of the International Association on Water Pollution Research* **50**, 83-90(2004).
8. Berry, D., Xi, C. & Raskin, L. Microbial ecology of drinking water distribution systems. *Current Opinion in Biotechnology* **17**, 297-302(2006).
9. Toze, S. et al. Inhibition of growth ofLegionella species by heterotrophic plate count bacteria isolated from chlorinated drinking water. *Current Microbiology* **21**, 139-143(1990).
10. Pace, N.R. A Molecular View of Microbial Diversity and the Biosphere. *Science* **276**, 734-740(1997).
11. Hoefel, D. et al. Culture-Independent Techniques for Rapid Detection of Bacteria Associated with Loss of Chloramine Residual in a Drinking Water System. *Appl. Environ. Microbiol.* **71**, 6479-6488(2005).
12. Kormas, K.A. et al. Changes of the bacterial assemblages throughout an urban drinking water distribution system. *Environmental Monitoring and Assessment* **165**, 27-38(2009).
13. Carter, J.T. et al. Relationships between levels of heterotrophic bacteria and water quality parameters in a drinking water distribution system. *Water Research* **34**, 1495-1502(2000).
14. Lavender, J.S. & Kinzelman, J.L. A cross comparison of QPCR to agar-based or defined substrate test methods for the determination of Escherichia coli and enterococci in municipal water quality monitoring programs. *Water Research* **43**, 4967-4979(2009).
15. Farnleitner, A.H. et al. Comparison and differentiation of heterotrophic plate count communities in raw and drinking water by eubacterial 16S-rDNA amplicon profiling. *Vom Wasser* **97**, 167-180(2001).
16. Farnleitner, A.H. et al. Eubacterial 16S-rDNA amplicon profiling: a rapid technique for comparison and differentiation of heterotrophic plate count communities from drinking water. *International journal of food microbiology* **92**, 333-45(2004).
17. Burtscher, M. et al. Heterotrophic plate count vs. in situ bacterial 16S rRNA gene amplicon profiles from drinking water reveal completely different communities with distinct spatial and temporal allocations in a distribution net. *Water SA (Online)* **35**, 495-504(2009).

18. Bent, S.J. & Forney, L.J. The tragedy of the uncommon: understanding limitations in the analysis of microbial diversity. *The ISME journal* **2**, 689-95(2008).
19. Allen, M.J., Edberg, S.C. & Reasoner, D.J. Heterotrophic plate count bacteria--what is their significance in drinking water? *International journal of food microbiology* **92**, 265-74(2004).
20. Eaton, A.D. et al. *Standard methods for the examination of water and wastewater*. (American Public Health Association: Washington, D.C., 2005).
21. Payment, P. et al. A prospective epidemiological study of gastrointestinal health effects due to the consumption of drinking water. *International Journal of Environmental Health Research* **7**, 5-31(1997).
22. Yarza, P. et al. The All-Species Living Tree project: a 16S rRNA-based phylogenetic tree of all sequenced type strains. *Systematic and applied microbiology* **31**, 241-50(2008).
23. Frank, D.N. XplorSeq: a software environment for integrated management and phylogenetic analysis of metagenomic sequence data. *BMC bioinformatics* **9**, 420(2008).
24. Pruesse, E. et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic acids research* **35**, 7188-96(2007).
25. Buchner, A. et al., with Ludwig, W. et al., Yadhukumar ARB: a software environment for sequence data. *Nucleic acids research* **32**, 1363-71(2004).
26. Hoefel, D. et al. Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. *Journal of Microbiological Methods* **55**, 585-597(2003).
27. Cohen, J. *Statistical power analysis for the behavioral sciences*. (Lawrence Erlbaum Associates: 1988).
28. Hughes, J.B. et al. MINIREVIEW Counting the Uncountable : Statistical Approaches to Estimating Microbial Diversity. *Society* **67**, 4399-4406(2001).
29. Grech, M., Carter, R. & Thomson, R. Clinical significance of Mycobacterium asiaticum isolates in Queensland, Australia. *Journal of clinical microbiology* **48**, 162-7(2010).